Hormone-Dependent Prostate Cancers are Dependent on Rac Signaling for Growth and Survival

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ABSTRACT

Prostate cancer remains a common cause of cancer mortality in men. Initially, cancers are dependent of androgens for growth and survival. First line therapies reduce levels of circulating androgens or target the androgen receptor (AR) directly. Although most patients show durable responses, many patients eventually progress to castration-resistant prostate cancer (CRPC) creating a need for alternative treatment options. The Rac1 signaling pathway has previously been implicated as a driver of cancer initiation and disease progression. We investigated the role of HACE1, the E3 ubiquitin ligase for Rac1, in prostate cancer and found that HACE1 is commonly lost resulting in hyperactive Rac signaling leading to enhanced cellular proliferation, motility and viability. Importantly, we show that a Rac inhibitor can attenuate the growth and survival of prostate cancer cells. Rac signaling was also found to be critical in prostate cancers that express the AR. Rac inhibition in androgen dependent cells resulted in reduction of AR target gene expression suggesting that targeting Rac1 may be an alternative method for blocking the AR signaling axis. Finally, when used in combination with AR antagonists, Rac inhibition enhanced the suppression of AR target gene expression. Therefore, targeting Rac in prostate cancer has the potential to enhance the efficacy of approved AR therapies.

Introduction

Prostate cancer is the most common cancer in men with over 170,000 estimated new cases in 2019 in the USA (1). Most newly diagnosed cases are characterized by slowly developing localized disease. More aggressive, rapidly growing forms of prostate cancer occur and can progress to metastatic disease. Therefore, better biomarkers capable of differentiating high-risk patients are desperately needed.

During initiation of prostate cancer, tumors depend on testosterone or the more potent dihydrotestostereone (DHT) for growth and survival (2). Androgens bind the androgen receptor (AR) in the cytoplasm. Once bound, the complex translocates into the nucleus where it binds DNA at androgen response elements (ARE; ref. 3). The AR complex promotes the transcription of androgen-regulated genes that control cellular growth, differentiation, and cellular survival. As such, prostate cancers are initially referred to as hormone (androgen) dependent cancers.

Rac1, a Rho family GTPase, has been implicated in driving a number of different types of cancers including breast, lung, colorectal, renal cell carcinoma, and others (4–8). Like other Rho family GTPases, Rac1 cycles between an inactive GDP-bound state and an activated GTP-bound state (9). Activated Rac1 signaling drives angiogenesis, cell growth, and survival as well as enhanced migration, invasion, and metastasis (7). In prostate cancer, Rac1 signaling has been associated with disease progression (10) and androgen-independent growth (11). VAV3, a Rac1 guanine exchange factor (GEF) that activates Rac1 is overexpressed in some prostate cancers potentiating AR transcriptional activity (12, 13). Elevated VAV3 levels also correlate with prostate cancer progression and posttreatment recurrence (14). Finally, expression of constitutively activated Rac1 has been implicated in ligand independent activation of AR transcriptional activity (13, 15).

To date, the prostate-specific antigen (PSA; ref. 16), is the most prevalently used biomarker for prostate cancer detection and prognosis (17). As the majority of prostate cancers are driven by the AR, treatment involves androgen deprivation therapy (ADT) that reduces circulating androgens. However, this approach eventually fails as tumors develop mechanisms of resistance to surgical or chemical castration resulting in castration-resistant prostate cancer (CRPC; ref. 18). The use of small molecule inhibitors that target the AR such as Enzalutamide have been shown to prolong survival in patients with CRPC disease (19). However, patients eventually develop resistance to these AR targeted agents. Therefore, better understanding of mechanisms of resistance along with better therapeutic strategies are needed.

In this study, we investigate the role of HACE1, the E3 ubiquitin ligase for activated Rac1 (4, 20), which is located at a chromosomal region that is commonly lost during progression to prostate cancer (21, 22). We show that HACE1 is commonly lost in prostate cancer progression resulting in hyperactivation of the Rac1 signaling pathway. Moreover, we show that blocking the Rac signaling pathway using a small molecule inhibitor attenuates growth and survival of prostate cancer cells. Interestingly, although Rac inhibition affected all prostate cancer cell lines screened, AR positive prostate cancer cells were more sensitive to Rac inhibition. We further demonstrate that AR transcriptional activity is dependent on activated Rac1 and that Rac inhibition blunts the ability of the AR to transcribe androgen regulated genes. This observation led to the characterization of a novel function of Rac1 as a critical AR transcriptional modulator and suggests that Rac inhibitors may be an alternative approach to target the AR signaling axis.

Material and Methods

Cell lines and cell culture

All cell lines were purchased from ATCC. HEK293T, DU145, and VCaP cells were cultured in DMEM. LNCaP cells were cultured in

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RPMI media. PC3 cells were cultured in F-12K media. Media were supplemented with 5% (v/v) FBS (Invitrogen). Mycoplasma testing (Lonza) were conducted every 5-cell passages. GYS32661 (23) was manufactured by Patheon. Enzalutamide was purchased from Selleckchem. Control and HACE1 viral transductions were conducted as described previously (4).

Cancer Genome Atlas data mining

The prostate adenocarcinoma dataset (PRAD) was accessed and mined through the Cancer Genome Atlas (PRAD) Research Network (Provisional 2017; http://cancergenome.nih.gov/).

Immunoblot assays

Cell lysates were prepared in RIPA lysis buffer. Blots were probed with Rac1 (1:1,000, Millipore, clone 23A8), HACE1 (1:1,000, Abcam, EPR7962), AR (1:1,000, Abcam, EPR1535; ref. 2), PSA (1:1,000 Abcam; EP1588Y) antibodies. Fluorescent tag anti-rabbit and anti-mouse secondary antibodies (Licor) were used. Signal was detected using Licor detection system. Blots were re-probed with an anti-actin (Abcam) antibody.

Rac pull-down experiments

Rac pull-down experiments were conducted as described previously (4).

Proliferation assay

A total of 1×10^3 cells were cultured in 6-well cell culture plates in triplicate. The cells were trypsinized and counted with hemocytometer for 3 to 6 consecutive days.

Migration assays

Cells were seeded in the upper compartment of a 24-well Boyden-Chamber (8-μm pore size; Costar) and allowed to migrate for 16 hours in response to complete media in the lower compartment. The cells that migrated to the underside of the filter were stained with crystal violet and counted under bright-field microscopy.

Soft-agar assay

The soft-agar colony-forming assay was performed in 6-well plates with a base of 2 mL of medium containing 5% FBS with 0.6% agar (Amresco). Cells were seeded in 2 mL of medium containing 5% FBS with 0.35% agar at 1×10^3 cells/well and layered onto the base. Two milliliters of media with 5% FBS was covered on the top of agar gel. The photographs of colonies growing in the plates were taken and scored using ImageJ.

Cytotoxicity assays

Cells were plated in 96-well plates (5,000 cells per well), and 24 hours later, various concentrations of GYS32661 were added and incubated for 3 days. Cytotoxicity was measured using a standard PrestoBlue dye (Invitrogen).

Xenograft assays

Adult (8–10 weeks of age) SCID mice were used for xenograft studies. A total of 1 × 106 VCaP cells were injected subcutaneously into the left flank of the mice. When the tumors reached approximately 100 to 150 mm3, mice were divided into treatment groups ($N = 5$). Saline was used as the vehicle control. Tumor volume and body weight were measured every 3 days. Tumor volume was calculated using the formula $V = (AB^2)/2$, where A is the largest diameter and B is the smallest diameter. Animal work was conducted under an approved IACUC protocol from the University of Miami Miller School of Medicine.

IHC

IHC staining for HACE1 was performed on prostate cancer tissue microarrays (US Biomax) as described previously (23). Each TMA spot was examined by two independent reviewers who assigned a score of 0 (no staining), 1 (<10% of malignant cells staining), 2 (10%–50% of malignant cells staining), or 3 (>50% of malignant cells staining) within carcinomatous areas. IHC staining of FFPE from tumors collected at experimental endpoint for PSA (Abcam, 1:100).

Statistical analysis

Data are expressed as mean ± SEM of $n = 3$ unless otherwise stated. Differences between 2 groups and multiple groups were analyzed by 2-tailed Student t test and 1-way ANOVA, respectively. P values less than 0.05 was considered significant. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

Results

HACE1 is commonly lost in prostate cancer resulting in hyperactivation of Rac1 signaling

Chromosomal deletions are thought to be early events in the transformation from normal to cancerous tissues. Comparative genomic hybridization (CHG) studies have identified regions frequently lost in prostate cancer including 6q16–22 that may contain putative tumor suppressor genes (24). We previously identified that HACE1, the E3 ubiquitin ligase for Rac1, is a tumor suppressor gene commonly lost in breast cancer located at chromosome 6q21 (4). To determine if HACE1 was lost in prostate cancer, HACE1 allelic fractions were investigated in the prostate adenocarcinoma dataset from The Cancer Genome Atlas (PRAD-TCGA) clinical dataset (**Fig. 1A**). Compared with blood and normal prostate, HACE1 DNA copy numbers are significantly decreased in prostate adenocarcinoma. To confirm that the loss of HACE1 alleles results in decreased expression of the HACE1 gene, HACE1 mRNA expression was also investigated in the PRAD-TCGA clinical dataset (**Fig. 1B**). Compared with the normal prostate, HACE1 expression was decreased in cancer tissues. In the same dataset, Rac1 DNA and mRNA levels were unchanged (Supplementary Fig. S1). To determine if lower HACE1 expression correlates with more aggressive disease states, we performed HACE1 IHC analysis on a panel of prostate cancer patient samples with varying stages of disease (**Fig. 1C**). HACE1 IHC analysis of prostate cancer patient samples confirmed that some tumors have reduced HACE1 protein levels. Although later stage disease had a trend towards the lowest HACE1 expression, only metastatic stage IV cancers reached significance (**Fig. 1D**). HACE1 analysis on a panel of prostate cancer cell lines indicated that HACE1 expression is lower than a representative normal cell line (**Fig. 1E**). Together, these data indicate that HACE1 loss is common during the transformation from normal prostate epithelium to prostate cancer.

HACE1 has previously been shown to lower levels of activated GTP-bound Rac1 through ubiquitin-mediated degradation (20). To determine if HACE1 controls activated Rac1 in prostate cancer cells, HACE1 was stably overexpressed in LnCaP cells and Rac1 activation assays were performed (**Fig. 2A**). Compared with empty vector (EV) control, HACE1 overexpression resulted in substantial reduction in active GTP-bound Rac1 whereas total Rac1 levels were unchanged. To ensure the regulation of active Rac1 by HACE1 is not dependent on the LnCaP cell line, Rac1 activation assays were also performed on VCaPs

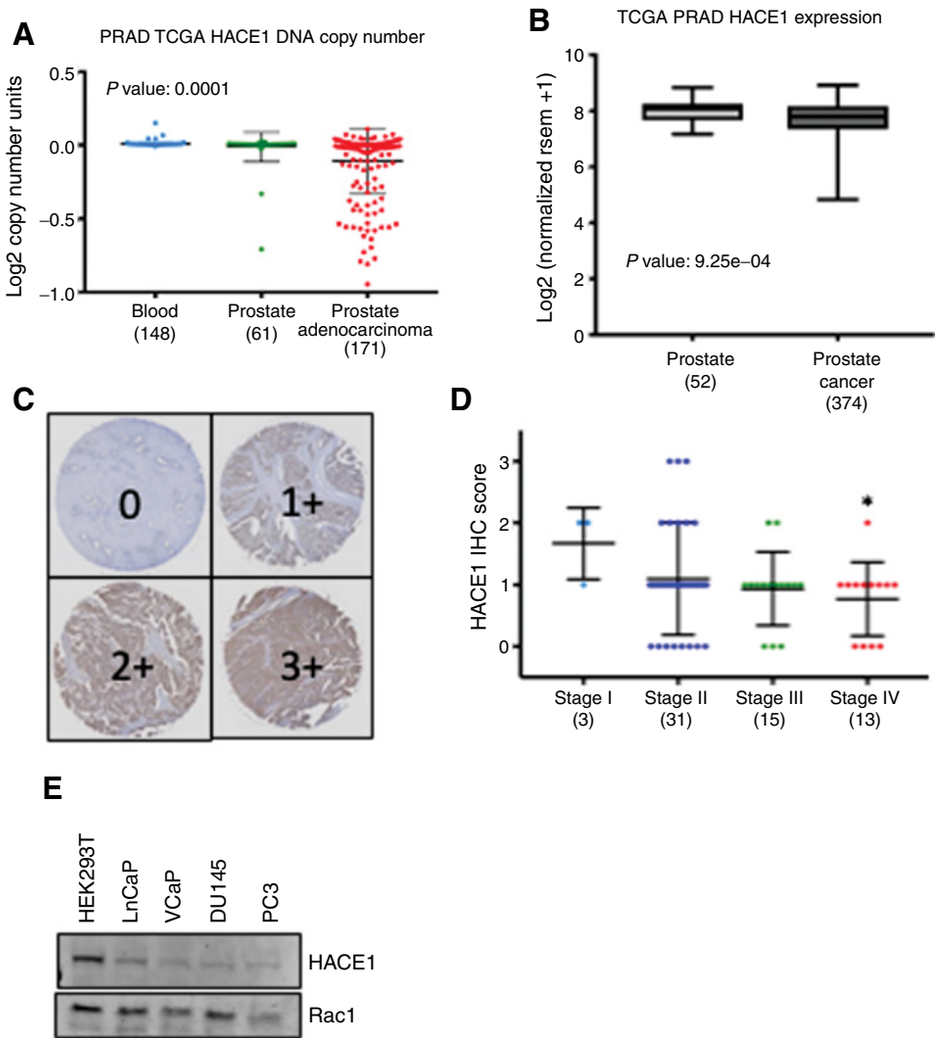

Figure 1.
HACE1 is lost during prostate cancer progression and correlates with poor prognosis. **A,** HACE1 DNA copy number analysis in normal blood, normal prostate, and prostate adenocarcinoma from the TCGA PRAD dataset. **B,** HACE1 mRNA expression in normal prostate and prostate adenocarcinoma from the TCGA PRAD dataset. **C,** Representative images of HACE1 IHC staining. **D,** HACE1 IHC scores from patients with prostate cancer by clinical stage. **E,** HACE1 protein levels in normal HEK293T and prostate cancer cell lines.

that overexpressed HACE1 (**Fig. 2B**). Similar to the LnCaP cell line, the overexpression of HACE1 resulted in reduction of active GTP-bound Rac1 whereas total Rac1 remained unchanged. Therefore, although HACE1 does not have an effect on total Rac1 levels, the pool of active GTP-bound Rac1 is severely diminished when HACE1 is present. Moreover, these data suggest that levels of HACE1 may infer Rac1 activity regardless of the amount of total Rac1 protein present.

In breast cancer cells, HACE1 overexpression resulted in attenuation of cellular proliferation (4). To determine HACE1 overexpression could affect the growth rates of prostate cancer cells, cellular proliferation assays were performed on control and HACE1 overexpressing LnCaP (**Fig. 2C**) and VCaP (**Fig. 2D**) cells. HACE1 overexpression resulted in significant impairment of prostate cancer cells to proliferate in 2D cell culture. LnCaP cells expressing a ligase dead HACE1 (C876S) showed no reduction in cellular growth indicating the growth suppressive effects of HACE1 overexpression are dependent on the protein's ability to ubiquitinylate Rac1 (**Fig. 2C**). Although the overexpression of HACE1 resulted in significant growth suppression, the knockdown of Rac1 resulted in an increase in cellular proliferation compared with controls (Supplementary Fig. S2). As Rac1 activity is canonically involved in cellular motility (25), we performed cellular migration assays on HACE1 overexpressing LnCaP (**Fig. 2E**) and VCaP (**Fig. 2F**) cells. HACE1 overexpression significantly blunted the migratory ability of both prostate cancer cell lines compared with the control cells. The ability of growth under anchorage-independent conditions has shown to be indicative of the growth potential of cancer cells in animals (26). Control and HACE1 overexpressing LnCaP cells were seeded in soft agar and the growth potential was assessed (**Fig. 2G**). Although the control LnCaPs displayed robust growth in soft agar, overexpression of HACE1 significantly impaired the ability of the cells to grow. Together, HACE1 expression in prostate cancer cell lines results in reduced levels of active Rac1 that translates to impaired migratory potential as well as growth in both 2D and 3D conditions.

Rac inhibition attenuates the growth and survival of prostate cancer cells

We previously characterized a novel Rac inhibitor that has been shown to be highly active in both colorectal cancer and renal cell carcinoma (8, 23). To determine if prostate cancer may be sensitive, cellular viability assays were performed on prostate cancer cell lines treated with Rac inhibitor GYS32661 (**Fig. 3A**). All cell lines had an IC_{50} at or below 10.5 μmol/L and were sensitive to Rac inhibition. Interestingly, prostate cancer cell lines that are androgen dependent

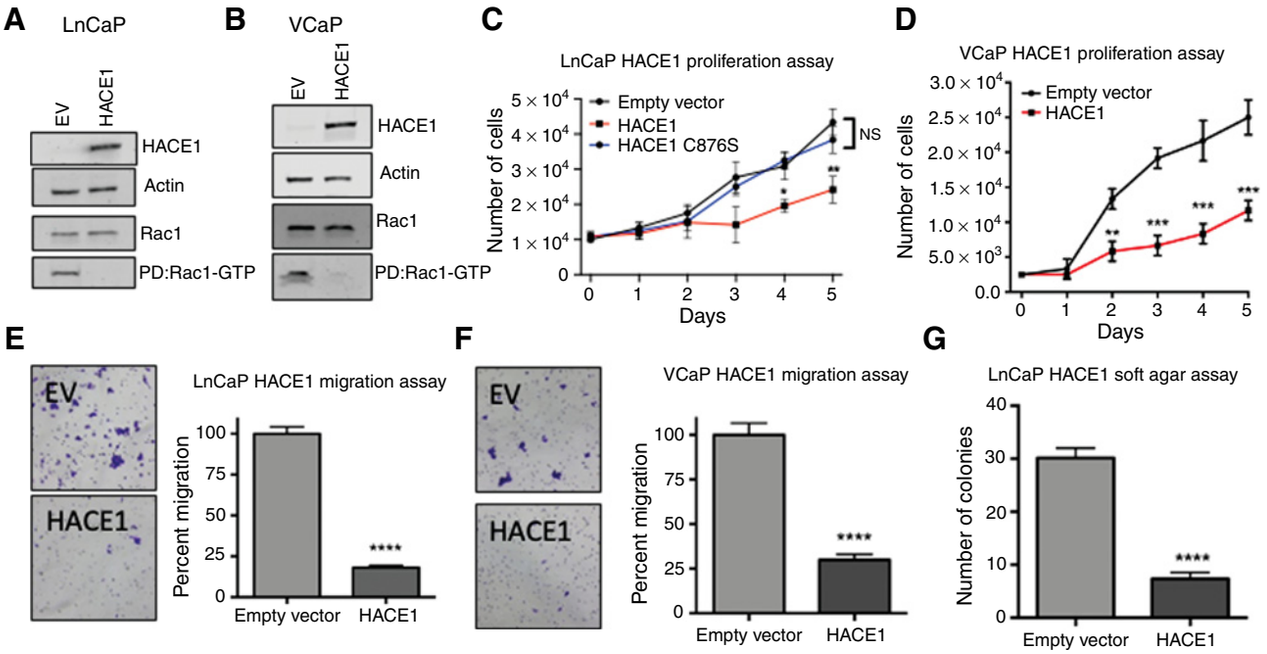

Figure 2.

HACE1 expression in prostate cancer reduces activated Rac1 levels. Rac1 GTP activation assays in HACE1 overexpressing (**A**) LNCaP and (**B**) VCaP prostate cancer cell lines. Whole cell lysate and Rac1-GTP immunoprecipitated with the GST-PBD were analyzed by Western blot analysis. Cellular proliferation assays of (**C**) LNCaP cells expressing HACE1 or HACE1 C876S and (**D**) VCaP cells expressing HACE1 or empty vector controls. Cell migration assays of (**E**) LNCaP and (**F**) VCaP cells expressing HACE1 or empty vector controls. **G,** Soft agar colony formation assay of LNCaP cells expressing HACE1 or empty vector controls.

were more sensitive than androgen-independent cells (**Fig. 3B**). To confirm Rac1 was inhibited by GYS32661, Rac1-GTP activation assays were performed on LnCaP cells (**Fig. 3C**). Rac1 inhibition was observed in a dose-dependent manner with levels of activated Rac1 significantly inhibited at concentrations as low as 5 μmol/L. GYS32661 also potently inhibited Rac1-GTP levels in the AR dependent VCaP (**Fig. 3D**) and androgen-independent PC3 (**Fig. 3E**) cells. Therefore, GYS32661 effectively inhibits active Rac1 in hormone dependent and independent prostate cancer cells at low micromolar concentrations. To confirm Rac inhibition decreased canonical Rac biological effects, cellular proliferation assays were performed on LnCaP cells in the presence of 2.5 and 5 μmol/L GYS32661 (**Fig. 3F**). Over 5 days of treatment, GYS32661 significantly impaired the ability of LnCaP cells to proliferate in a dose-dependent manner. To confirm the anti-proliferative effects of GYS32661 were mediated by Rac1 inhibition, proliferation assays were also conducted on VCaP and LnCap cells that were transfected with siRac1 or a nonsilencing control (Supplementary Fig. S3). Rac1 molecular knockdown phenocopied the anti-proliferative effects of GYS3261 treatment. Anchorage-independent growth potential of LnCaP cells were also tested in the presence of increasing concentration of GYS32661 (**Fig. 3G**). Similar to results from the 2D proliferation assay, treatment with GYS32661 significantly diminished the ability of the LnCaP cells to grow in soft agar. Finally, we determined the effects of GYS32661 on cell migration. As expected, GYS32661 significantly impaired the migratory ability of androgen dependent LnCaP, and androgen independent PC3, and DU145 cells (**Fig. 3H** and **I**; Supplementary Fig. S4A) to migrate in a Boyden chamber assay.

It was previously reported that the overexpression of constitutively active Rac1 enhanced ERK1/2 activation in androgen-dependent LnCaP cells (13). To investigate the effects of Rac inhibition on intracellular signaling pathways, an unbiased phosphor-kinase array was conducted on LnCaP cells treated with either vehicle or GYS32661 (**Fig. 3J**; Supplementary Fig. S4B). Although activated ERK1/2 was not observed in either condition, a dramatic reduction in phospo-AKT and one of its downstream kinases, GSK3β was observed upon treatment with the Rac inhibitor. Together, these data indicate the Rac inhibitor GYS32661 blocks the AKT signaling pathway and can significantly impair growth, migratory potential, and cellular viability of prostate cancer cells at low micromolar concentrations *in vitro*.

Rac inhibition blocks AR transcriptional activity

Treatment of the Rac inhibitor on a panel of prostate cancer cells indicated that cells expressing AR were more sensitive to Rac inhibition, suggesting there might be an association between Rac1 and AR (**Fig. 2A**). To determine if Rac1 inhibition had an effect on AR activity, expression of PSA, a canonical AR target gene (16), was investigated in VCaP cells treated with GYS32661 (**Fig. 4A**). Enzalutamide, a clinically employed AR antagonist was used as a positive control. Stimulation of VCaP cells with DHT resulted in potent induction of PSA gene expression, whereas treatment with DHT in the presence of Enzalutamide significantly reduced PSA gene induction indicating inhibition of AR transcriptional activity. VCaP cells treated with DHT in the presence GYS32661 also repressed PSA expression comparable to levels of Enzalutamide treated cells. GYS32661 also inhibited DHT induced PSA expression in LNCaPs, indicating this phenomenon is not cell specific (Supplementary Fig. S5A). To further investigate AR transcriptional activity, VCaP cells were stably transduced with an AR response element (ARE) luciferase reporter (**Fig. 4B**; ref. 27). Upon DHT binding to the AR, the AR transcriptional complex binds ARE

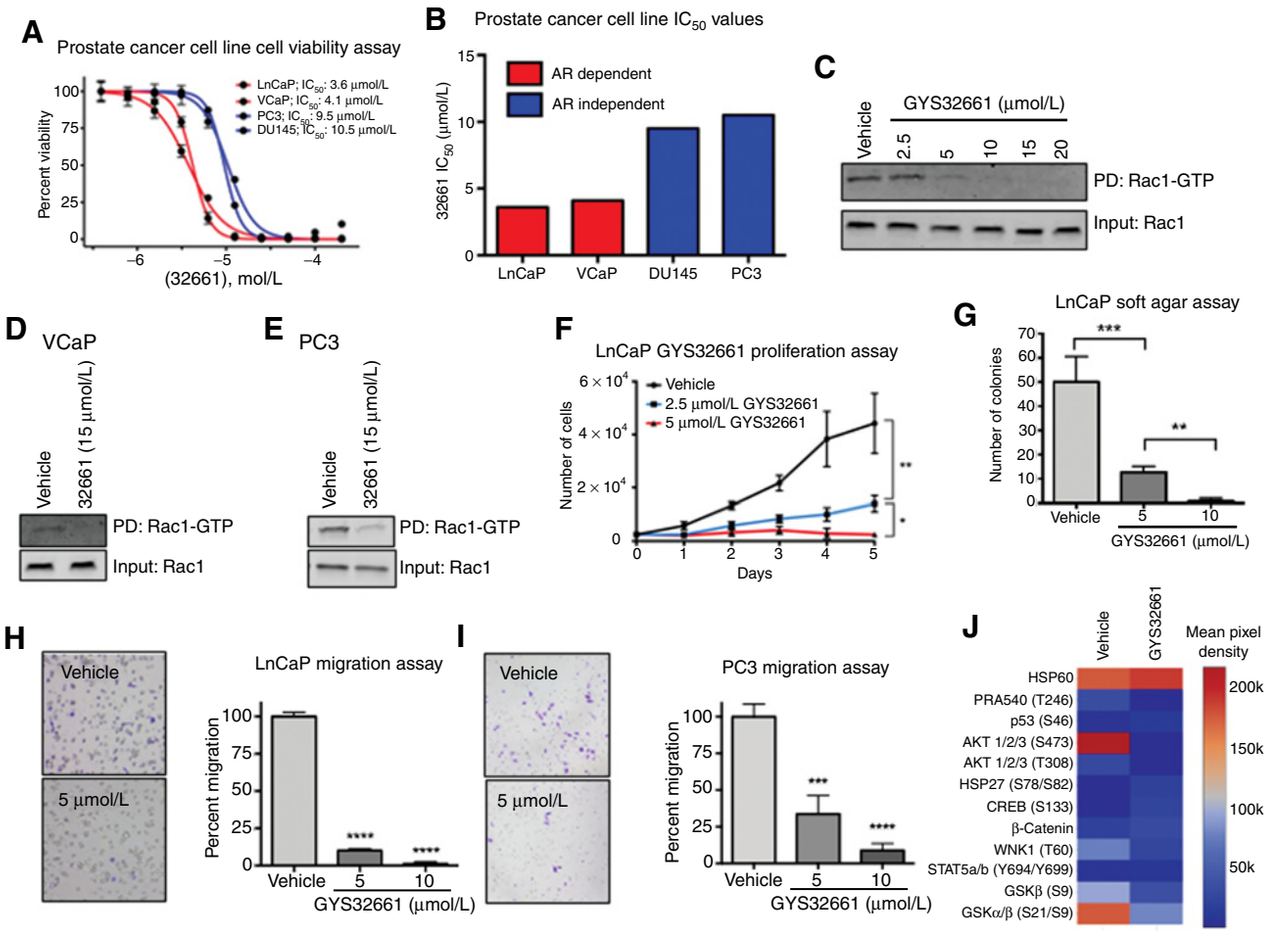

Figure 3.

Rac inhibition in prostate cancer. **A,** Cellular viability assays on a panel of prostate cancer cell lines treated with increasing doses of GYS32661 (**B**) IC_{50} values for GYS32661 for prostate cancer cell line panel. **C,** Rac1 GTP activation assays in LNCaP cells treated with increasing concentrations of GYS32661. Whole cell lysate (WCL) and Rac1-GTP immunoprecipitated with the GST-PBD were analyzed by Western blot analysis. Rac1 GTP activation assays on (**D**) VCaP and (**E**) PC3 cells treated with GYS32661. WCL and Rac1-GTP immunoprecipitated with the GST-PBD were analyzed by Western blot analysis. **F,** Cellular proliferation assay on LNCaP cells treated with Vehicle or GYS32661. **G,** Soft agar colony formation assay on LNCaP cells treated with Vehicle or GYS32661. **H,** Migration assay on (**H**) LNCaP and (**I**) VCaP cells treated with vehicle or GYS32661. Left: Representative images; right panel: quantification of cellular migration. **J,** Phospho-kinase RPPA analysis of LNCaP cells treated with vehicle control or 10 μmol/L GYS32661.

reporter sites and induces luciferase expression that are read as relative luciferase units (RLU). The addition of DHT to VCaP cells induces a potent luciferase signal. As expected, introduction of DHT in the presence of Enzalutamide significantly suppressed the activity of the ARE luciferase signal in a dose-dependent manner. More impressively, the DHT stimulation in the presence of the Rac inhibitor resulted a dose-dependent inhibition of the ARE luciferase signal to an even greater extent than the Enzalutamide at comparable doses. The same effect was also observed in LnCaP cells, suggesting that the effects are not cell type specific (Supplementary Fig. S5B). The molecular knockdown of Rac1 using siRNA also suppressed AR transcriptional activity confirming the effects are mediated through inhibition of Rac1 (Supplementary Fig. S5C). Finally, to confirm the effects observed in decreased AR transcriptional activity resulted in decreased protein expression of AR target genes, VCaP cells treated with 10 nmol/L DHT in the presence of Enzalutamide or GYS32661 were analyzed by Western blot analysis (**Fig. 4C**). DHT stimulation of VCaPs resulted in increased PSA protein expression. PSA protein levels however, were significantly reduced in the presence of Enzalutamide. The Rac inhibitor also suppressed PSA protein levels. Again, these results were observed in LnCaP cells indicating that the effects are not limited to a single cell line (Supplementary Fig. S5D). Molecular knockdown of Rac1 also suppressed the ability of VCaP cells to induce PSA levels upon stimulation with DHT (Supplementary Fig. S5E). Interestingly, Rac inhibition also appeared to reduce total levels of AR protein, suggesting an additional element of control of the AR signaling axis. To further investigate how Rac inhibition may be controlling AR activity, cellular fractionations were performed on VCaP cells stimulated with DHT in the presence or absence of GYS32661 (**Fig. 4D**). In the absence of DHT, the majority of AR and Rac1 pools reside in the cytoplasm. No significant changes in AR or Rac1 are observed when the cells are treated with the Rac inhibitor without DHT stimulation. As previously reported, DHT stimulation results in the AR translocating from the cytoplasm to the nucleus (28). Interestingly, a shift of Rac1 was also observed from the cytoplasm to the nucleus upon DHT stimulation. Both AR and Rac1 translocation were observed in VCaP cells

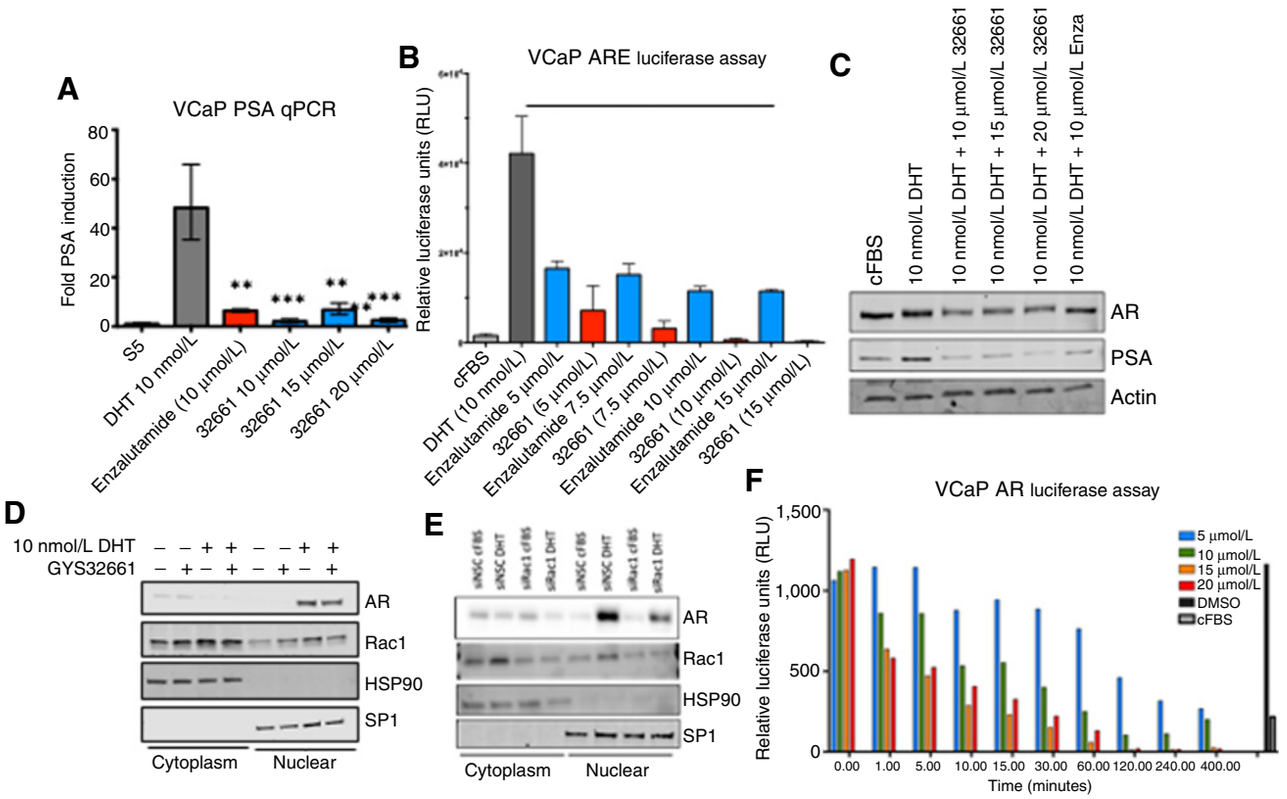

Figure 4.

Rac inhibition blocks AR transcription *in vitro*. **A,** PSA RT-PCR from VCaP cells stimulated with 10 nmol/L DHT in the presence of Enalutamide or GYS32661. **B,** ARE Luciferase reporter assay on VCaP Cells stimulated with 10 nmol/L DHT in the presence of Enzalutamide or GYS32661. **C,** Western blot analysis of VCaP cells stimulated with 10 nmol/L DHT in the presence of Enzalutamide or GYS32661. **D,** Cellular fractionation assay on VCaP cells stimulated with 10 nmol/L DHT in the presence of GYS32661. Lysates were analyzed by Western blot analysis. **E,** Cellular fractionation assay on siNSC and siRac1 VCaP cells stimulated with 10 nmol/L DHT in the presence of GYS32661. Lysates were analyzed by Western blot analysis. **F,** VCaP ARE luciferase assay time course of cells stimulated with 10 nmol/L DHT. At the indicated time point, media was removed and replaced with cFBS containing media. All conditions were analyzed at 480 minutes after stimulation.

stimulated with DHT in the presence of the GYS32661. However, the amount of AR protein that translocated from the cytoplasm to the nucleus was less in Rac inhibitor treated cells. To further investigate this observation, cellular fractionation experiments were conducted on DHT stimulated VCaP cells that were transfected with either control or Rac1-targeted siRNA (**Fig. 4E**). Similar to cells treated with the Rac inhibitor, VCaP cells that had Rac1 knocked down showed less AR translocation to the nucleus.

To investigate how durable the suppressive effects of Rac inhibition has on AR, we performed an experiment where VCaP cells expressing the ARE luciferase reporter were pulsed with DHT and the varying concentrations of the Rac inhibitor for different time intervals and then washed out (**Fig. 4F**). After 480 minutes, luciferase readings were taken. Control cells that were incubated with 10 nmol/L DHT showed a significant increase in luciferase signal compared with control cells that were unstimulated. Cells that were treated with all concentrations of GYS32661 for 0.02 minutes and then the drug was washed out saw no reduction in luciferase signal. However, exposure to 10 μmol/L of GYS32661 and higher for only 1 minute resulted in a measurable decrement in luciferase signal. The reduction in luciferase signal increased as both exposure time and drug concentration increased in a dose-dependent manner where concentrations of GYS32661 of 15 μmol/L for a 10-minute exposure period resulted in reduction of the luciferase signal that was comparable to the signal observed in untreated cells. These data suggest that a durable response of AR target gene suppression can be achieved with short exposure times to the Rac inhibitor.

In vivo inhibitory effects of the Rac inhibitor were explored in VCaP cells inoculated into immunocompromised mice. Once tumors reached 150 mm^3, animals were randomized into vehicle control, enzaluatmide, and GYS32661 treatment arms (**Fig. 5A**). The tumors from vehicle-treated animals grew rapidly with the first animal reaching endpoint on treatment day 20. Tumors from Enzalutamide-treated mice showed a significant reduction in tumor growth compared with the vehicle group with the first animal reaching endpoint on experimental day 25. Tumors from animals treated with the Rac inhibitor showed even greater reduction of tumor growth compared with both the vehicle and the Enzalutamide treatment groups with the first animal reaching experimental endpoint on day 38. Kaplan–Meier survival curves of the animals in each of the experimental arms indicate that treatment with GYS32661 significantly prolonged the survival of the animals compared with the vehicle- and Enzalutamide-treated arms (**Fig. 5B**). To determine if the suppression of AR in the VCaPs also occurs *in vivo*, tumor sections were stained for PSA using IHC (**Fig. 5C**). Vehicle-treated tumors had strong PSA staining whereas both the Enzalutmide and GYS32661 tumors showed a marked

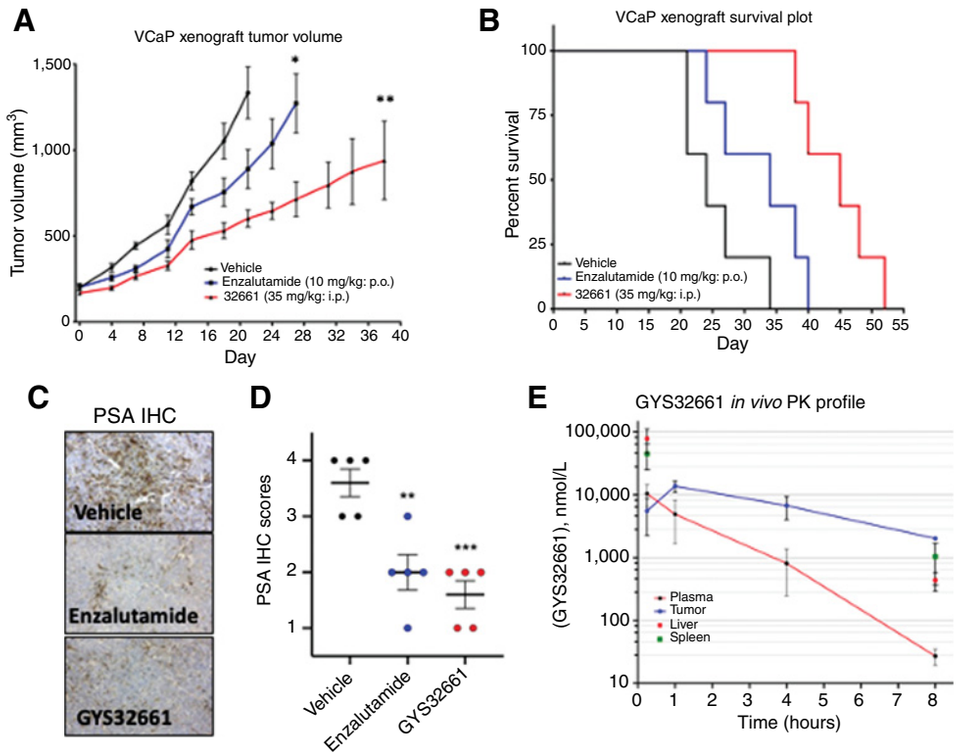

Figure 5.
Rac inhibition blocks AR transcription *in vivo*. **A,** NSG mice inoculated with VCaP tumor xenografts were treated with either vehicle, Enzalutamide (10 mg/kg, p.o.) or GYS32661 (35 mg/kg, i.p.). **B,** Kaplan–Maier survival curves of VCaP tumor bearing mice treated with Vehicle, Enzalutamide, or GYS32661. **C,** Representative PSA IHC staining of Vehicle, Enzalutamide, and GYS32661 treated tumors. **D,** PSA IHC tumor scores for Vehicle, Enzalutamide, and GYS32661 treated tumors. **E,** GYS32661 pharmacokinetic analysis of plasma, tumor, liver, and spleen from VCaP tumor bearing mice treated with 35 mg/kg GYS32661.

reduction in PSA levels confirming that disruption of the AR signaling axis was blocked *in vivo* (**Fig. 5D**). To obtain a pharmacokinetic (PK) profile of GYS32661 in the animals as well as the amount of drug that reached the tissues, concentrations of GYS32661 were measured in plasma, tumors, as well as liver and spleen for comparison (**Fig. 5E**). At 15 minutes after drug administration, plasma concentrations of GYS32661 reached levels higher than 10 μmol/L with a terminal half-life of 0.81 hours. Maximum exposure levels of drug in the spleen and liver were even higher. Interestingly, at the 15-minute time point, intratumoral concentrations of GYS32661 were 4.5 μmol/L and peaked at the 1-hour time point where concentrations were found to be 13.7 μmol/L. The GYS32661 also seemed to have longer intratumoral retention where concentrations remained in the micromolar range for at least 8 hours. On the basis of the results from the *in vitro* studies, intratumoral concentrations of GYS32661 that are capable of suppressing AR target gene expression are achieved for prolonged periods.

Rac inhibition enhances the effects of AR antagonists in blocking AR transcriptional activity

As it was determined that Rac inhibition was capable of attenuating AR transcriptional activity, we wanted to determine if the combination of the Rac inhibitor with an agent that directly targets AR such as enzalutmide could enhanced AR signaling blockade. To test this experimentally, VCaP cells stimulated with DHT were treated with Enzalutamide, GYS32661, or the combination of the two drugs and PSA gene expression was investigated (**Fig. 6A**). DHT treatment resulted in significant expression of PSA mRNA. As previously observed, both the single agent enzalutamide and GYS32661 blocked the PSA mRNA expression. However, when used together, the two agents significantly decreased expression of PSA compared with either agent alone in an additive manner. We also investigated the combinatorial drug effects in the ARE luciferase reporter assay (**Fig. 6B**). Again, DHT treatment induced a strong luciferase signal that was reduced when treated with Enzalutamide or GYS32661. Similar to PSA mRNA expression, the combination of Enzalutamide plus GYS32661 resulted in significant suppression of the luciferase signal compared with either agent alone. To further investigate the extent of the combinatorial approach, dose titrations of both Enzalutamide and GYS32661 were used in combination in the ARE luciferase assay (**Fig. 6C**). Dose-dependent suppression of the luciferase signal was observed when either Enzalutamide or GYS32661 was used in combination. To confirm the mRNA and luciferase assay observations resulted in reduced protein levels of AR target genes, we investigated the combination treatment in LNCaP cells. DHT treatment results in elevated PSA protein levels whereas the treatment of the LNCaPs with either Enzalutamide or GYS32661 reduced PSA levels. The combination of Enzalutamide and GYS32661 in the LNCaP cells resulted in even greater suppression of PSA at the protein level. Finally, to determine if the improvements in blocking AR activity were seen *in vivo*, VCaP tumor bearing mice were treated with vehicle control, enzalutamide or GYS32661 alone, or the combination of Enzalutamide and GYS32661 (**Fig. 6E**). A dose of 35 mg/kg of GYS32661 reduced the tumor growth to rates comparable with 20 mg/kg of Enzalutamide. When used in combination, the two drugs completely blocked the ability of the VCaP tumors to grow *in vivo*. Therefore, although Enzalutamide and GYS32661 have the ability to suppress AR signaling as single agents, the combinational approach yields significantly better blockade of the AR signaling axis.

Discussion

Although better understanding of the underlying mechanisms of prostate cancer have resulted in improvements in patient outcomes, it

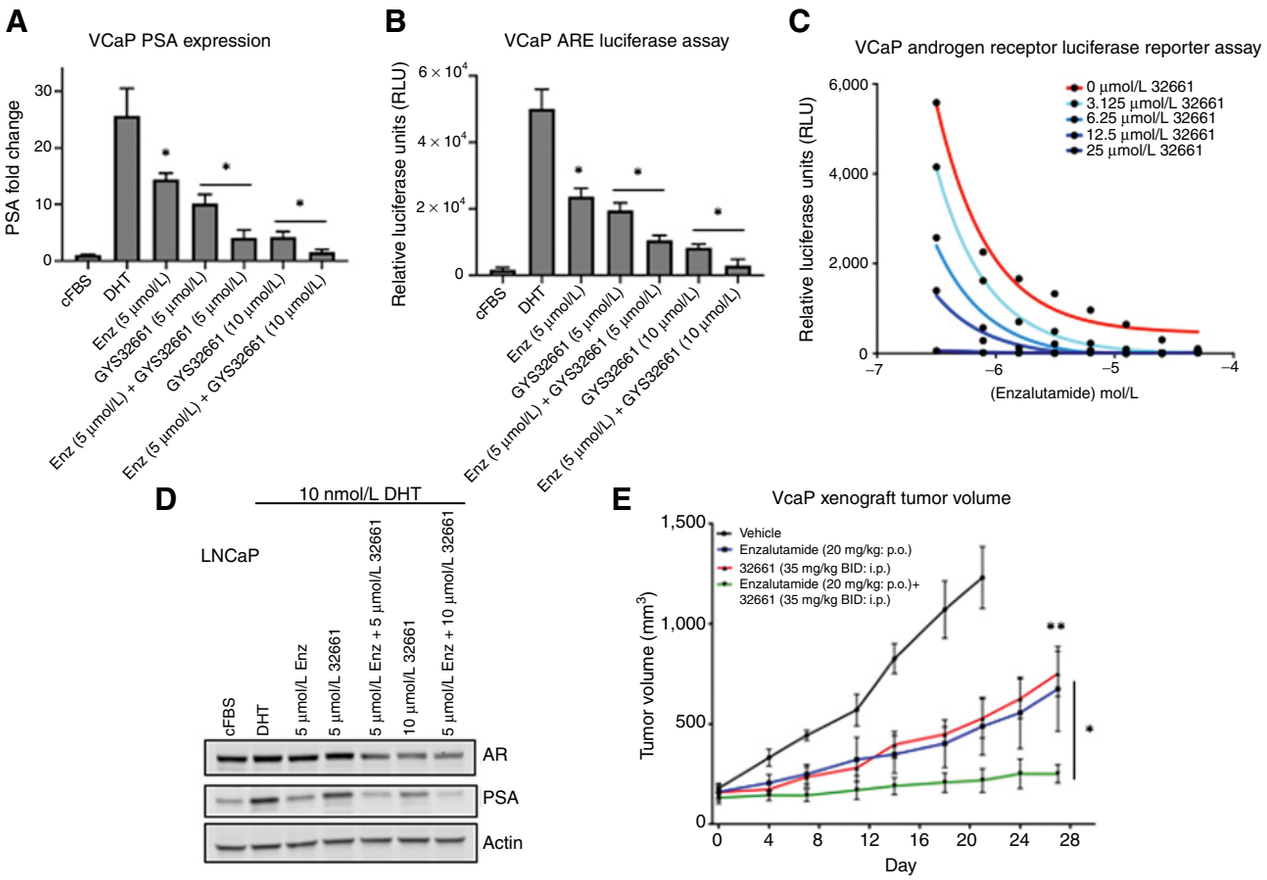

Figure 6.

Rac inhibition improves the effects of Enzalutamide. **A,** PSA RT-PCR from VCaP cells stimulated with 10 nmol/L DHT in the presence of Enalutamide, GYS32661, or the combination of Enzalutamide and GYS32661. **B,** ARE Luciferase reporter assay on VCaP Cells stimulated with 10 nmol/L DHT in the presence of Enalutamide, GYS32661, or the combination of Enzalutamide and GYS32661. **C,** ARE Luciferase reporter assay on VCaP Cells treated with the combination of Enzalutamide and GYS32661. **D,** Western blot analysis of LNCaP cells stimulated with 10 nmol/L DHT in the presence of Enalutamide, GYS32661, or the combination of Enzalutamide and GYS32661. **E,** NSG mice inoculated with VCaP tumor Xenografts were treated with vehicle, Enzalutamide (20 mg/kg, p.o.), GYS32661 (35 mg/kg, i.p.), or the combination of Enzalutamide and GYS32661.

is estimated that over 30,000 patients will die of their disease (1). Although most prostate cancers initially respond to castration techniques (29), resistance eventually develops with tumor progression despite low androgen levels (30). Resistance mechanisms circumvent low levels of circulating androgens such as overexpression of the AR or intracrine androgen production. Upstream activation of growth factor receptor signaling has been shown to cross-regulate AR signaling (31). Overexpression of the Rac1 GEF VAV3 has been shown to activate AR transcriptional activity under androgen independent conditions (13). The overexpression of constitutively activated Rac1 in androgen-dependent prostate cancer cells also results in androgen-independent activation of AR transcriptionally activity through a mechanism that is mediated through activation of the MAPK signaling pathway (13, 15). Activating posttranslational modifications of AR have also been suggested to confer activation of the receptor in the absence of ligand (32). It is now appreciated that many CRPCs remain dependent on AR for growth and survival. This continued dependency of AR is exemplified by the improved outcomes of patients receiving therapeutics that target AR and block ligand binding such as Enzalutamide (33). Unfortunately, resistance eventually occurs though multiple mechanisms including mutations in the ligand binding domain that prevent drug binding to the mutant receptors (34). As such alternative approaches to targeting AR are needed.

In this study, we investigated the role of Rac1 signaling in prostate cancer. Unlike other types of cancers, Rac1 overexpression was not observed in prostate cancer (Supplementary Fig. S1B). However, previous reports of VAV3, a Rac GEF, have shown that VAV3 is overexpressed in prostate cancers leading to activation of Rac1 (14). In the same study, it was shown that ectopic expression of constitutively activated Rac1 resulted in ligand independent activation of AR transcriptional activity that was mediated by activation of the MAPK signaling pathway. In this study, we identified that Rac inhibition resulted in suppression of the AKT signaling pathway in a model of androgen-dependent prostate cancer.

In addition to overexpression of proteins that activate Rac1, we also identified that HACE1, the E3 ubiquitin ligase for activated Rac1, is commonly lost in prostate cancer. HACE1 allelic loss has been reported in breast and other cancers (4, 20, 21, 35–38). HACE1 promoter methylation has also been observed colorectal cancer, gastric cancer, and hepatocellular carcinoma (39–41). The loss of HACE1 results in the accumulation activated Rac1 driving downstream Rac

signaling that results in enhanced proliferation, cell survival, and migration (36, 42, 43). Although it appears that Rac1 is not overexpressed, higher levels of Rac activating GEFs in combination with HACE1 loss result in hyperactivation of the Rac signaling pathway in prostate cancer. HACE1 overexpression in prostate cancer cells that express low protein levels resulted in reduced Rac activation resulting in decreased cellular proliferation, anchorage-independent growth, and cellular migration. Therefore, reducing the levels of activated Rac1 may have profound effects on tumor growth and progression.

Rac1 is a proto-oncogene that has been implicated in driving many different types of cancers making it an interesting drug target (7, 44). A few Rac inhibitors such as NSC23766 and EHT1864 (45, 46) have been previously been shown to have impressive *in vitro* activity. AZA1, a dual Rac/CDC42 inhibitor has previously been shown to suppress the growth of prostate cancer cells *in vivo* (47). Unfortunately, poor chemical properties have limited their *in vivo* use making them unsuitable for clinical development. GYS32661 is a novel Rac inhibitor designed to have better *in vivo* activity (23). Here, we show that GYS32661 is highly effective in attenuating the *in vitro* and *in vivo* growth and survival of prostate cancer cells. By treating a panel of representative cell lines, we discovered that prostate cancer cells dependent on AR appeared to be more sensitive to Rac inhibition than cell lines that are AR independent. This observation led to the characterization that Rac activity appears to be critical for AR translocation into the nucleus as well as AR transcriptional activity. The dependency of AR on Rac activation suggests that Rac inhibition may be an alternative approach to blocking the AR signaling axis in prostate cancer. However, additional studies are also necessary to elucidate the mechanism of how Rac interacts with AR and mediate its transcriptional activity. Temporal studies where cell lines were exposed to drug for limited durations showed prolonged suppression of AR target gene transcriptional activity. This observation may explain why a durable antitumor response occurs when the plasma half-life of GYS32661 is less than 1 hour. However, it is also noteworthy that GYS32661 was retained in the prostate tumors whereas plasma concentrations were cleared. These observations suggest that further pharmacodynamics studies are critical to better understand the *in vivo* utility of GYS32661.

These findings also uncovered another important observation regarding the control of AR signaling. We observed that Rac inhibition was capable of enhancing the suppressive effects of AR targeted therapies such as Enzalutamide. Patients that fail first-line AR targeted therapies appear to remain sensitive on AR signaling for survival (48). Therefore, once patients become refractory to initial AR LBD targeted therapies, the addition of a Rac inhibitor may circumvent escape mechanisms.

In summary, we identified that HACE1, an E3 ubiquitin ligase for Rac1, is commonly lost during the progression of normal epithelium to prostate cancer. As in other types of cancer, HACE1 loss results in hyperactivation of the Rac signaling pathway resulting in enhanced proliferation, migration, and viability. Rac-driven prostate cancers are highly sensitive to a small molecule Rac inhibitor both *in vitro* and *in vivo*. We found that Rac1 is an important mediator of AR transcriptional activity and that Rac inhibition may be an alternative approach to targeting the AR axis in prostate cancer.

Authors' Disclosures

E.T. Goka reports other from Geneyus LLC outside the submitted work; and has a patent for US10,272,352 B2 issued. D.T. Mesa Lopez reports other from Geneyus LLC outside the submitted work. M.E. Lippman reports other from Geneyus LLC during the conduct of the study; other from Geneyus LLC outside the submitted work; and also has a patent for US 10,272,352 B2 issued.

Authors' Contributions

E.T. Goka: Conceptualization, resources, formal analysis, supervision, validation, writing–original draft, writing–review and editing. **D.T. Mesa Lopez:** Conceptualization, resources, data curation, formal analysis, investigation. **M.E. Lippman:** Conceptualization, supervision, writing–original draft, project administration, writing–review and editing.

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